Modified ubiquitin regulatory system

Field of the Invention

This present invention relates in general to gene expression and is in particular concerned with regulatory systems for regulating gene expression based on the ubiquitin regulatory system (URS) and the use of these regulatory systems in combination with an expressible structural gene, preferably a plant expressible structural gene, for the regulated expression of said structural gene and for a regulated expression control when stressed for instance with elevated temperature.

Background to the Invention

Genetic engineering of plants

The hurdle of creating successful genetically engineered plants in major crop varieties is now being overcome sequentially on a plant-by-plant basis. The term "genetic engineering" is meant to describe the manipulation of the genome of a plant, typically by the introduction of a foreign gene into a plant, or the modification of the genes of the plant, to increase or decrease the synthesis of gene products in the plant. Typically, genes are introduced into one or more plant cells which can be cultured into whole, sexually competent, viable plants which may be totally transformed or which may be chimeric, having some tissues transformed and some not. These plants can be self-pollinated or cross-pollinated with other plants of the same or compatible species so that the foreign gene or genes carried in the germ line can be bred into agriculturally useful plant varieties.

Current strategies directed toward the genetic engineering of plant lines typically involve two complementary processes. The first process involves the genetic transformation of one or more plant cells of a specifically characterized type. The term "transformation" as used herein means that a foreign gene, typically in the form of a genetic construction, is introduced into the genome of the individual plant cells.

This introduction is accomplished through the aid of a vector, which is integrated into the genome of the plant. The second process then involves the regeneration of the transformed plant cells into whole sexually competent plants. Neither the transformation nor regeneration process need to be 100% successful, but must have a reasonable degree of reliability and reproducibility so that a reasonable percentage of the cells can be transformed and regenerated into whole plants.

EP-A-0342926 (the content of which is incorporated herein by reference) discloses a plant (maize) ubiquitin regulatory system comprising a heatshock element (comprising two overlapping consensus heatshock elements), a promoter, a transcription start site, an intron and a translation start site. The heatshock element component of this regulatory system is believed to confer heat inducibility of expression of associated DNA sequences in dicot or monocot cells following permissive levels of heatshock.

Plant ubiquitin regulatory system refers to the approximately 2 kb nucleotide sequence 5' to the translation start site of the ubiquitin gene and comprises sequences that direct initiation of transcription, control of expression level, induction of stress genes and enhancement of expression in response to stress. The regulatory system, comprising both promoter (of about 1 kb nucleotide sequence) and regulatory functions, is the DNA sequence providing regulatory control or modulation of gene expression. A structural gene placed under the regulatory control of the plant ubiquitin regulatory system means that a structural gene is positioned such that the regulated expression of the gene is controlled by the sequences comprising the ubiquitin regulatory system.

Promoters are DNA elements that direct the transcription of RNA in cells. Together with other regulatory elements that specify tissue and temporal specificity of gene expression, promoters control the development of organisms.

There has been a concerted effort in identifying and isolating promoters from a wide variety of plants and animals, especially for those promoters demonstrating a high

level of constitutive expression and capable of maintaining stable levels of said expression under stress conditions.

The present invention is based on modifications of the plant ubiquitin regulatory system.

Summary of the Invention

In one aspect the present invention provides a DNA sequence comprising a ubiquitin regulatory system lacking heatshock elements.

Because the ubiquitin regulatory system lacks heatshock elements, it is not heat inducible.

In a further aspect the invention thus provides a DNA sequence comprising a ubiquitin regulatory system that is not heat inducible substantially comprising the nucleotide sequence according to SEQ.ID.NO.8.

For brevity the ubiquitin regulatory system forming part of a DNA sequence in accordance with either of these aspects of the invention will be referred to as a modified ubiquitin regulatory system (mURS).

The mURS preferably substantially comprises a plant URS, such as a maize URS e.g. as disclosed in EP-A-0342926. The term "substantially comprises" in this context means that the mURS corresponds generally to an unmodified URS other than of course in regions where the mURS is modified, e.g. by lacking heatshock elements.

The mURS may thus comprise an intron, e.g. as disclosed in EP-A-0342926.

An mURS may be produced, e.g., by modification of an URS by removal of one or more heatshock elements therefrom, e.g. using standard DNA manipulation techniques well known to those skilled in the art.

In a further aspect the invention provides a DNA construct comprising a DNA sequence in accordance with the invention and a plant-expressible structural gene under the regulatory control of the ubiquitin regulatory system of said sequence.

The invention also provides an expression vector comprising such a DNA construct.

The mURS of the invention may be used in analogous manner as the URS described in EP-A-0342926, and reference is herewith made to that document for further details. In particular, the mURS can be used to regulate expression of an associated structural gene in cells, particularly plant cells (monocot or dicot).

The invention thus covers use of a DNA sequence, DNA construct or expression vector in accordance with the invention for transforming cells, particularly plant cells.

A further aspect of the invention provides a method of transforming a host cell, particularly a plant cell, comprising introducing into the cell a DNA sequence, DNA construct or expression vector in accordance with the invention.

Methods for achieving such transformation are well known to those skilled in the art and basically comprises the steps of constructing a plant expression vector that comprises a protein-encoding sequence and the modified ubiquitin regulatory system according to the invention and introducing the expression vector into a plant cell.

Preferably the plant cell is propagated into a plant and the protein-encoding sequence is expressed. The present invention is also a transgenic plant cell, plant and seed comprising a gene construct comprising the modified ubiquitin regulatory system.

Said plant is preferably a monocot such as wheat, barley, oat, corn or maize. Most preferably it is wheat.

The invention thus also includes within its scope a host cell, particularly a plant cell, into which has been introduced a DNA sequence, DNA construct or expression vector in accordance with the invention.

The invention further provides a method of expressing a structural gene in a host cell in a constitutive manner, the method comprising the steps of: causing to be present in the host cell the structural gene, operably linked to a DNA sequence in accordance with the invention defined above and causing the structural gene to be expressed constitutively.

The modified ubiquitin regulatory system or the promoter may be truncated to determine the smallest fragment capable of expression. Methods of truncating include deleting sequences and digesting the sequence with a restriction enzyme or other nuclease with the purpose of remaining substantially the same property and/or activity as the untruncated sequence. These methods are commonly known in the art of molecular biology.

To assess promoter activity usually a transient reporter gene expression system is used. In such a system or assay, the fragment to be assayed would be linked to a reporter gene and used to transform a plant cell. Useful reporter genes include chloramphenicol acetyltransferase (CAT), luciferase (Lux) and β-glucuronidase (GUS).

The mURS of the invention functions in generally the same way as an unmodified URS except that it is not inducible in response to heat (and possibly also in response to other conditions of stress). The invention thus provides a novel regulatory system which can confer non-heat-inducible constitutive expression of associated DNA sequences. The advantage of this system is that the expression of associated DNA sequences that it mediates in transformed plant cells is stable and

not influenced by environmental changes in temperature which would normally affect expression mediated by a non-modified system e.g. as described in EP-A-0342926.

The mURS has been shown to function to give high levels of constitutive expression, comparable to those obtainable from non-modified (wild-type) URS, and to be capable of maintaining stable levels of constitutive expression under conditions of heat stress.

EP-A-0342926 includes definitions of various terms that are used in the present specification, including "expression", "promoter", "regulatory control", "structural gene", "plant ubiquitin regulatory system", "heatshock elements", and "introns" and those definitions also apply to these terms when used in the present specification.

The invention will be further described, by way of illustration, in the following Examples and with reference to the accompanying drawings and Tables as well, in which:

Figure 1 is a restriction map of plasmid pPBI96-36;

Figure 2 is a restriction map of plasmid pdHUbiGUS;

Figure 3 shows the predicted sequence of the mURS sequence in pPBI97-U3, with the KpnI site which replaces the overlapping heatshock elements in the wild-type URS being boxed (this Figure corresponds to SEQ.ID.NO.8);

Figure 4 is a restriction map of plasmid pPBI97-dUG1;

Figure 5 is a restriction map of plasmid pPBI97-2BdUN1.

Figure 6 shows the restriction map of plasmid pUN1 which contains the wild type URS driving the NptII selectable marker gene.

Figure 7 is a bar chart showing the mean relative level of NptII expression from each transformation event after heat shock (grey) and without heat shock (black). Results from lines transgenic for the wild type URS are shown in panel (a) and results from the mURS are shown in panel (b).

Figure 8 is a schematic illustration of a particle bombardment chamber (not to scale)

Tables 1 and 2 show the level of NptII expression in each plant (expressed relative to the rRNA control) with and without heat shock treatment and the transformation event from which the plants were derived.

EXAMPLES

Example 1

Investigation of effect on expression of removing the heatshock elements from the ubiquitin regulatory system

Two overlapping consensus heatshock (HS) elements in the maize ubiquitin regulatory system (URS) are defined in EP 0342926 and US 5,614,399. A modified URS (mURS) was produced as described below.

The plasmid pPBI95-1 is a derivative of pAHC25 (Christensen, AH & Quail, PH 1996. Transgenic Research 5:213-218) in which a SacI linker sequence [d(pCGAGCTCG)] (New England Biolabs [NEB] catalogue no. 1044) has been inserted at the SmaI site of pAHC25.

A mURS lacking the heatshock elements was constructed from two PCR fragments which were amplified using pPBI95-1 as template using the following primer combinations.

GUS 1 : 5'TCGCGATCCAGACTGAATGCC 3' (SEQ ID No: 1) with
HS1: 5' ATTA**GGTACC**GGACTTGCTCCGCTGTCGGC (SEQ ID No: 2).
and
HS2: 5' TATA**GGTACC**GAGGCAGCGACAGAGATGCC 3' (SEQ ID No: 3) with
Ubi5': 5' ATATGCTGCAGTGCCAGCGTGACCCGG 3' (SEQ ID No: 4).

GUS1 + HS1 amplify a fragment of approximately 1330bp. The resulting fragment has a KpnI site (from primer HS 1) and a SacI site (from pPBI95-1) close to its 5' and 3' ends respectively. Ubi5' + HS2 amplify a fragment of approximately 680bp. The resulting fragment has a PstI site (from pPBI95-1) and a KpnI site (from primer HS 2) close to its 5' and 3' ends respectively.

The resulting GUS1/HS1 and Ubi5'/HS2 amplified fragments were digested with KpnI and SacI and with KpnI and PstI respectively and double ligated into the PstI and SacI sites of pUC19. The resulting re-constituted mURS was then transferred as a HindIII/SacI fragment, replacing the non-modified URS in a plasmid pPBI96-36 (Figure 1) to produce the plasmid pdHUbiGUS (Figure 2). The plasmid pPBI96-36 comprises the GUS-Nos reporter gene fusion under the control of the wild-type ubiquitin promoter (derived from pAHC25) in a pUC plasmid backbone.

The primer design is such that a 32bp sequence (TGGACCCCTCTCGAGAGTTCCGCTCCACCGTT) (SEQ ID No: 5) containing the two overlapping consensus heatshock elements in the URS defined in US 5,614,399 are replaced by a KpnI (GGTACC) site in the mURS.

The ability of the mURS to mediate high levels of expression of an associated DNA sequence was tested in transient GUS expression analyses by particle bombardment of pdHUbiGUS and pPBI96-36 into wheat and barley immature embryos. pPBI96-36 is identical to pdHUbiGUS except that it comprises the wild-type URS rather than the mURS. Both constructs gave rise to high levels of GUS expression as visualised by observing the number and intensity of blue foci visualised following histochemical analysis using X-gluc (methods as described in

Jefferson RA [1987] Assaying chimaeric genes in plants: The GUS gene fusion system. Plant Molecular Biology Reporter 5 (4) 387-405). In fact the GUS expression mediated by the two constructs was essentially indistinguishable.

Example 2

Amplification of a mURS using maize genomic DNA as template

A second mURS was prepared via PCR amplification of two DNA fragments using maize genomic DNA (maize genotype B73) as template, followed by ligation of the two fragments to produce a single fragment lacking the consensus heatshock (HS) elements. Again a KpnI restriction site was engineered in place of the HS elements.

The PCR primers used were designed from sequence information published by Liu et al 1995 (Biochem Cell Biol 73: 19-30; database accession ZMU29159). To delete the HS element from the wild-type URS and to replace it with a diagnostic KpnI site two fragments were amplified using the primer combinations HS1 + Ubi3-3 and HS2 + Ubi5-2, the sequences of which are given below. Primers Ubi5-2 and Ubi3-3 are homologous to sequences in the promoter sequence published by Liu et al. Primers HS1 and HS2 are homologous to sequences located immediately 3' and 5' respectively of the two overlapping HS elements in the ubiquitin promoter as discussed above. Both of these primers have a KpnI tail (shown in bold in the sequences) at their 5' ends.

HS1: 5- ATTA**GGTACC**GGACTTGCTCCGCTGTCGGC –3 (SEQ ID No: 2)
HS2: 5- TATA**GGTACC**GAGGCAGCGACAGAGATGCC – 3 (SEQ ID No: 3)
Ubi5-2: 5- AGCTGAATCCGGCGGCATGGC – 3 (SEQ ID No: 6)
Ubi3-3: 5- TGATAGTCTTGCCAGTCAGGG – 3 (SEQ ID No: 7)

The amplified products were subcloned into pGEM TEasy (Promega) to produce the plasmids pPBI97-U1 and pPBI97-U2. Appropriate orientations for subsequent

subcloning were determined by restriction digest analysis. A full-length (2Kb) mURS sequence including the promoter and intron was reconstructed by subcloning a KpnI – SacI fragment from pPBI97-U1 into the KpnI/SacI sites of pPBI97-U2 to produce pPBI97-U3. The predicted sequence of the cloned mURS fragment in pPBI97-U3 is presented in Figure 3 as SEQ ID No: 8. The KpnI site which replaces the overlapping heatshock elements in the wild-type URS is boxed. pPBI97-U3 contains approximately 35bp of sequence at its 5' end and approximately 40bp of sequence at its 3' end, none of which is present in the plasmid pAHC25 or its derivatives.

The mURS was transferred as a PstI fragment from pPBI97-U3 into the PstI sites of pPBI96-36 replacing the wild-type URS in pPBI96-36 to produce pPBI97-dUG1 (sometimes also referred to as p97-dUG1) (Figure 4). The orientation of the modified promoter was determined using the KpnI site which is present in the modified but not wild-type promoter. pPBI96-36 and pPBI97-dUG1 are identical except that pPBI96-36 contains the wild-type URS from pAHC25 whereas pPBI97-dUG1 contains the mURS from plasmid pPBI97-U3.

The function of the mURS in pPBI97-dUG1 was confirmed by transient transformation analyses by particle bombardment into various plant tissues and comparison with the expression mediated by the wild-type URS in pPBI96-36.

The following plant tissues were analysed: wheat and barley immature embryos, wheat leaves, wheat roots, tobacco leaves, oil palm cell suspensions.

Following bombardment the tissues were incubated at 20°C for 24 hours prior to histochemical analysis.

The results as visualised by GUS expression were indistinguishable between the two different plasmids, indicating that deleting the heatshock sequence does not affect the capacity of the modified promoter to mediate high levels of constitutive expression in these tissues under these conditions.

Example 3

The maize genome-derived mURS in pPBI97-dUG1 has also been transferred upstream of a neomycin phosphotransferase (NptII) sequence to produce a plasmid pPBI97-2BdUN1 (sometimes also referred to as P97-2BdUN1) (Figure 5). This plasmid has been used successfully as a selectable marker construct in the stable transformation of wheat, as described in European Patent Application No. 98307337.0, and repeated hereafter.

Example 4

The mURS confers non-heat-inducible constitutive expression.

Plant Transformation

Immature embryos (IMEs) of the wheat variety Bob White were bombarded with pPBI97 2BdUN1 which comprised the mURS driving the NptII selectable marker gene. In independent experiments, IMEs were also bombarded with plasmid pUN1 (Figure 6) which comprised the wild type URS driving NptII.

A number of independent primary transformants (Ro generation) were produced.

Heat shock treatment.

A total of five events transformed with pPBI97 2BdUN1 and two events transformed with pUN1 were selected for analysis of heat inducibility. Primary transformants were allowed to set seed and the R1 seed was collected. Between 22 and 25 R1 seeds per independent event were planted and seedlings were tested for NptII activity via leaf bleach assay. A total of 8 - 12 NptII leaf bleach assay positive plants from each original event were selected and grown in a glasshouse to the 2-3 leaf stage. Plants were then removed from the glasshouse and 4-6 plants

from each event were heat shocked for 2 hours at 42 degrees C in a Vulcan™ incubator, while 4-6 plants from each event were left at room temperature, i.e. non heat shocked. Leaf material was harvested from all lines, both heat shocked and non heat shocked, and stored at –70°C prior to analysis.

RNA Isolation and Northern Blotting

Frozen leaf tissue from each plant was ground to a fine powder under liquid nitrogen in a Braun Mikrodismembrator™. Total RNA was extracted from approximately 100 mg frozen ground tissue using the Qiagen Rneasy™ extraction kit according to manufacturers instructions. 15 µg of total RNA was electrophoresed on a 1% agarose, 2.21M formaldehyde, 40mM MOPS pH7.0, 10 mM sodium acetate, 1 mM EDTA gel, in a 40 mM MOPS pH 7, 10 mM sodium acetate, 1 mM EDTA running buffer at 1 V/cm overnight. Gels were washed briefly in sterile distilled H_2O, and blotted onto HyBond N^+ (Amersham International), according to standard protocols (Sambrook et al, 1989) overnight. Blots were then dismantled and airdried for 2 hours, before UV fixing at 312 nm for 2 minutes.

Probe Labelling and Hybridization

25 ng of the appropriate probe (NptII, or wheat ribosomal 25S fragment) were radiolabelled using the Rediprime 11 ™ system (Amersham International) using α ^{32}PdCTP (Amersham International) according to manufacturers instructions. Blots were hybridized overnight at 65°C in 0.6M NaCl, 20mM Pipes, 4mM $Na_2EDTA.2H_20$, 0.2% gelatin, 0.2% Ficoll400, 0.2% PVP-360, 10mM $Na_4P_2O_7.10H_20$, 0.8% SDS, 0.5 mg/ml denatured salmon sperm DNA. Post hybridization washes were carried out in 30mM NaCl, 2Mm $NaH_2PO_4.2H_2O$, 0.2 mM $Na_2EDTA.2H_2O$, 0.1% SDS at room temperature for 30 minutes, then 65° for 10 minutes. Blots were exposed to Typhoon™ General Purpose phosphorimager screens for 1-2 days depending on signal strength, and the screens were scanned on the Typhoon™ Phosphorimager to quantitate signal intensity.

The NptII expression was determined relative to the ribosomal-RNA level in order to standardise variation in total RNA loading.

Results.

The relative expression of NptII in progeny from two independent events (lines 694 and 695) transformed with pUN1 (wild type URS) is shown (Table 1). The mean level of expression in progeny from line 694 after heat shock was 5X higher than in progeny maintained at room temperature (Fig. 7a). Similarly, expression in progeny from line 695 showed a 3.4X induction after heat shock (Fig. 7a). This confirms that the wild type URS is heat inducible.

The relative expression of NptII in progeny from five independent events (lines 563, 564, 578, 604, 618) transformed with pPBI97 2BdUN1 (mURS) is shown (Table 2). In all lines, the mean level of expression after heat shock was either less than or approximately equal to that in plants maintained at room temperature indicating that expression from the mURS is not heat inducible (Fig. 7b). This demonstrates that removal of the heat shock elements from the URS leads to a non-heat inducible pattern of expression.

Table 1.

	Heat Shock			Room Temp		
	Plant number	Relative NptII expression	Mean	Plant number	Relative NptII expression	Mean
Wild Type URS						
Line 694	1	3.38	**7.30**	14	1.22	**1.44**
	2	6.78		10	1.27	
	3	9.67		11	2.23	
	5	6.39		12	1.03	
	6	10.3				
Line 695	1	1.32	**1.43**	2	0.47	**0.42**
	4	1.4		5	0.38	
	12	0.83		9	0.32	
	13	0.72		10	0.47	
	7	2.88		23	0.47	

Table 2

	Plant number	Heat Shock Relative Nptll expression	Mean	Plant number	Room Temp Relative Nptll expression	Mean
Modified URS						
Line 563	1	0.28	**0.48**	12	0.61	**0.61**
	11	0.30		13	0.51	
	6	0.44		14	0.57	
	7	0.44		15	0.27	
	8	0.92		16	1.1	
Line 564	1	0.93	**1.17**	3	1.06	**1.32**
	7	1.92		4	1.34	
	9	1.06		5	1.24	
	10	0.88		19	1.15	
	16	1.04		23	1.82	
Line 578	12	1.14	**0.94**	3	0.87	**0.84**
	13	1.31		4	0.66	
	14	0.9		6	1.02	
	18	0.61		7	0.88	
	19	0.72		21	0.75	
Line 604	1	0.91	**0.47**	8	0.91	**1.14**
	2	0.12		10	1.64	
	3	0.1		11	1.21	
	4	0.45		18	0.78	
	16	0.77				
Line 618	2	0.28	**0.32**	10	1.12	**0.71**
	3	0.44		11	0.65	
	15	0.3		14	0.47	
	16	0.4		6	0.73	
	18	0.24		8	0.85	
	19	0.23		9	0.42	

Methods and Materials used in the examples described above

The wheat transformation method used and described here is largely based on the method disclosed by Barcelo and Lazzeri (1995): Transformation of cereals by microprojectile bombardment of immature inflorescence and scutellum tissues; Methods in Molecular Biology-Plant Gene Transfer and Expression Protocols (vol 49), 113-123; Jones H (ed) Humana Press Inc., Totowa, NJ.

Embryo wheat plants of the spring cultivar Bob White were grown in a glasshouse with 16hr day length supplemented with lights to maintain a minimum light intensity of 500 μmol $m^{-2}s^{-1}$ at 0.5M above flag leaf. Glasshouse temperatures were maintained at 19°C+/-1°C during the day and 14°C+/-1°C at night.

Immature embryos of wheat were harvested from developing grain. The seeds were harvested and embryos were cultured at approximately 12 days after anthesis when the embryos were approximately 1mm in length. Seeds were first rinsed in 70% ethanol for 5 minutes and then sterilised in a 10% solution of Domestos bleach (Domestos is a Trade Mark) for 15 minutes followed by 6 washes with sterile distilled water. Following removal of the embryonic axis the embryos were placed axis surface face down on agargel (Sigma catalogue no. A-3301) solidified MM1 media. The general recipe for MM1 is given in Appendix 1, and the recipes for the various constituents in Appendix 2. The embryos were maintained in darkness for one to two days at 24°C +/-1°C prior to bombardment.

The plasmids pUN1 and p97-2BdUN1 were used to provide selection markers. The plasmids pUN1 and p97-2BdUN1 contain chimeric promoter-NptII gene fusions and provide selection of transformants against a range of aminoglycoside antibiotics including kanamycin, neomycin, geneticin and paromycin.

Particle bombardment was used to introduce plasmids into plant cells. The following method was used to precipitate plasmid DNA onto 0.6μm gold particles

(BIO-RAD catalogue number 165-2262): A total of 5µg of plasmid DNA was added to a 50µl - sonicated for one minute - suspension of gold particles (10mg/ml) in a 1.5ml microfuge tube. Following a brief vortex for three seconds 50µl of a 0.5M solution of calcium chloride and 20µl of a 0.05M solution of spermidine free base were added to the opposite sides of the microfuge tube lid. The tube contents were mixed together by closing the lid and tapping the calcium chloride and spermidine to the bottom of the tube. Following a vortex for three seconds the suspension was centrifuged at 13,000 rpm for 5 seconds. The supernatant was then removed and the pellet resuspended in 150µl of absolute ethanol. This requires scraping the gold particles off the inside of the tube using a pipette tip. Following a further three second vortex, the sample was centrifuged again and the pellet resuspended in a total volume of 85µl in absolute ethanol. The particles were vortexed briefly and sonicated for 5 seconds in a Camlab Trisonic T310 water bath sonicator to ensure fine dispersion. An aliquot of 5µl of the DNA coated gold particles were placed in the centre of a macrocarrier (BIO-RAD catalogue no. 115-2335) and allowed to dry for 30 mins. Particle bombardment was performed by using a Biolisitc™ PDS-1000/He (BIO-RAD Instruments, Hercules CA) chamber which is illustrated schematically in Figure 8, using helium pressure of 650 and 900 psi (rupture discs: BIO-RAD catalogue numbers 165-2327 and 165-2328 respectively).

Referring to Figure 8, the illustrated vacuum chamber comprises a housing 10, the inner side walls of which include a series of recesses 12 for receiving shelves such as sample shelf 14 shown at the fourth level down from the top of the housing. A rupture disc 16 is supported in a He pressure shock tube 18 near the top of the housing. A support 20, resting in the second set of recesses 12 down from the top of the housing, carries unit 22 that includes a stopping screen and a number of rings 24, with 11 rings below the support 20 and 3-4 rings above the support 20. Macrocarrier 26 is supported at the top of unit 22. The approximate distance from the rupture disc 16 to the macrocarrier 26 is 25mm, with the approximate distance from the macrocarrier 26 to the stopping screen being 7mm, and the approximate distance from the stopping screen to the sample shelf 14 being 67mm. The top of unit 22 is about 21mm from the bottom of the shock tube 18, and the bottom unit 22

is about 31mm from the top of sample shelf 14.

Immature embryos were bombarded between 1 and 2 days after culture. For bombardment the immature embryos were grouped into a circular area of approximately 1cm in diameter comprising 20-100 embryos, axis side face down on the MM1 media. A petri dish containing the tissue was placed in the chamber on shelf 14, on the fourth shelf level down from the top, as illustrated in Figure 8. The air in the chamber was then evacuated to a vacuum of 28.5 inches of Hg. The macrocarrier 26 was accelerated with a helium shock wave using rupture membranes that burst when the He pressure in the shock tube 18 reaches 650 or 900 psi. Within 1 hour after bombardment the bombarded embryos were plated on MM1 media at 10 embryos per 9cm petri dish and then maintained in constant darkness at 24°C for 2-3 weeks. During this period somatic embryogenic callus was produced on the bombarded embryos.

After 2-3 weeks the embryos were transferred onto agar-solidified regeneration media, known as R media, and incubated under 16hr day length at 24° C. The general recipe for R media is given in Appendix 1. Embryos were transferred on fresh plates at 2-3 week intervals. For selection of transformants using the NptII gene three different regimes were used: 1) Geneticin (GIBCO-BRL catalogue no. 10131-019) was incorporated (at 50mg/L) immediately on transfer to regeneration media and maintained at 50mg/L on subsequent transfers to regeneration media. 2) & 3) Embryos were first transferred to regeneration media without selection for 12 days and 2-3 weeks, respectively, and thereafter transferred on to media containing Geneticin at 50mg/L. After 2-3 passages on regeneration media regenerating shoots were transferred to individual culture tubes containing 15 ml of regeneration media at half salt strength with selection at 35mg/L geneticin. Following root formation the regenerated plants were transferred to soil and the glass house.

Leaf bleach assay

Primary transformants and progeny were confirmed as transgenic by leaf bleach

assay as described in Plant Physiol. (1997) 115: 971-980. Leaf pieces were vacuum infiltrated with paromomycin and scored for resistance after 2-3 days. This method was validated by comparison with results from analysis of genomic DNA via Southern blotting.

Genomic DNA isolation and Southern Analyses

Southern analyses of primary transformants and progeny material were carried out as follows: Freeze dried leaf tissues were ground briefly in a Kontes™ pestle and mortar, and genomic DNA extracted as described in Fulton et al, 1995. 5 µg of DNA were digested with an appropriate restriction enzyme according to the manufacturers instructions, and electrophoresed overnight on a 1% agarose gel, after which the gel was then photographed, washed and blotted onto Hybond N+™ (Amersham International) according to the method of Southern using standard procedures (Sambrook et al 1989, Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbour Press, Cold Spring Harbour, NY). Following blotting, the filters were air dried, baked at 65°C for 1-2 hours and UV fixed at 312nm for 2 minutes.

Probe preparation and labelling for the Southern analyses of transformed material was carried out as described above.

GUS histochemistry was performed essentially as described in Jefferson (1987), Plant Molecular Biology Reporter,5,(4),387-405.

Appendix 1.

Recipe for 2x concentrated MM1 media

Constituent	Volume of stock per litre of 2x concentrated media
Macrosalts MS (10X stock)	200ml
Microsalts L (1000x stock)	2ml
FeNaEDTA MS (100x stock) [Sigma catalogue F-0518]	20ml
Modified Vits MS (x1000)	1ml
3 amino acid solution (25x stock)	40ml
myo inositol (Sigma catalogue number I-3011)	0.2g
sucrose	180g
$AgNO_3$ (20mg/ml stock) Added after filter sterilisation	1ml
Picloram (1m/ml stock) Added after filter sterilisation	4ml

Filter sterilise and add to an equal volume of moulten 2x agargel (10g/L).

Recipe for 2x concentrated R media

Constituent	Volume of stock per litre of 2x concentrated media
Macrosalts L7 (10X stock)	200ml
Microsalts L (1000x stock)	2ml
FeNaEDTA MS (100x stock)	20ml
Vits/Inositol L2 (200x stock)	10ml
3 amino acid solution (25x stock)	40ml
Maltose	60g
2,4-D (1mg/ml stock) added after filter sterilisation	200µl
Zeatin cis trans mixed isomers (Melford labs catalogue no. Z-0917) (5mg/ml stock) added after filter sterilisation	2ml

Filter sterilise and add to an equal volume of moulten 2x agar (16g/litre)

Appendix 2

Recipes for constituents of MM1 and R media

Microsalts L (1000x stock)

	per 100ml
$MnSO_4.7H_2O$	1.34g
H_3BO_3	0.5g
$ZnSO_4.7H_2O$	0.75g
KI	75mg
$Na_2MoO_4.2H_2O$	25mg
$CuSO_4.5H_2O$	2.5mg
$CoCl_2.6H_2O$	2.5mg

Filter sterilise through a 22µm membrane filter
Store at 4°C

Macrosalts MS (10X stock)

	per litre
NH_4NO_3	16.5g
KNO_3	19.0g
KH_2PO_4	1.7g
$MgSO_4.7H_2O$	3.7g
$CaCl_2.2H_2O$	4.4g

NB: Dissolve $CaCl_2$ before mixing with other components
NB: Make up KH_2PO_4 separately in sterile H_20, and add last.
Store solution at 4°C after autoclaving

Modified MS Vits (1000x stock)

	Per 100ml
Thiamine HCl	10mg
Pyridoxine HCl	50mg
Nicotinic acid	50mg

Store solution in 10ml aliquots at -20°C

3 amino acid solution (25x stock)

	Per litre
L-Glutamine	18.75g
L-Proline	3.75g
L-Asparagine	2.5g

Store solution in 40ml aliquots at -20°C

Macrosalts L7 (10x stock)

	per litre
NH_4NO_3	2.5g
KNO_3	15.0g
KH_2PO_4	2.0g
$MgSO_4.7H_2O$	3.5g
$CaCl_2.2H_2O$	4.5g

NB: Dissolve $CaCl_2$ before mixing with other components

NB: Make up KH_2PO_4 separately in 50ml H_20 and add last

Store solution at 4°C after autoclaving

Vits/Inositol (200x stock)

200x Stock	Per 100ml
Inositol	4.0g
Thiamine HCl	0.2g
Pyridoxine HCl	0.02g
Nicotinic acid	0.02g
Ca-pantothenate	0.02g
Ascorbic acid	0.02g

Store solution in 40ml aliquots at -20°C